# Dual Column Chromatographic Method for Determination of *N*-Nitrosothiazolidine in Fried Bacon

JOHN W. PENSABENE and WALTER FIDDLER
*Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA 19118*

**A dual column chromatographic procedure is described that permits the determination of *N*-nitrosothiazolidine (NTHZ), a newly identified nitrosamine, and *N*-nitrosopyrrolidine (NPYR), the principal volatile nitrosamine, in the same fried bacon sample. The procedure used an acid-Celite, Celite, sodium sulfate column followed by a partially deactivated basic alumina column to isolate NTHZ from bacon for subsequent quantitation by gas chromatography with a thermal energy analyzer (GC-TEA). A limited intralaboratory study gave standard deviations for repeatability and reproducibility of 1.20 and 1.55 ppb, respectively. The recovery of *N*-nitrosothiomorpholine, the internal standard added at the 10 ppb level, was 93.3 ± 6.03%. The method is also free from artifactual nitrosamine formation.**

We recently reported the previously unidentified nitrosamine, *N*-nitrosothiazolidine (NTHZ), in fried bacon (1). The amount of NTHZ in bacon is questionable, however, because a general method for its determination is not available. Initially, NTHZ was isolated using extracts from the mineral oil distillation procedure developed by Fine et al. (2). However, the use of this procedure can result in nitrosamine artifacts, especially when residual sodium nitrite is present in fried bacon before analysis (3). The FDA multidetection procedure used (4) for the isolation of volatile nitrosamines was not suitable because NTHZ is not steam-distillable. Therefore, a direct extraction procedure, one that does not result in artifactual nitrosamine formation, was needed to measure NTHZ. We recently reported a rapid screening procedure for the isolation and detection of *N*-nitrosopyrrolidine (NPYR) in fried cure-pumped bacon, using a column chromatographic–thermal energy analyzer technique, which did not result in apparent artifactual nitrosamine formation (3). This method was modified by an additional column chromatographic step to enable the quantitative determination of NTHZ and NPYR from a single fried bacon sample.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Received January 27, 1982. Accepted March 23, 1982.

## METHOD

Use caution when handling nitrosamines because they are potential carcinogens.

### *Apparatus*

(a) *Mortar and pestle.*—Glass, 473 mL (16 oz), A. H. Thomas Co.

(b) *Chromatographic column.*—Glass, 350 mm long × 32 mm id with 60 mm long × 6 mm id drip tip.

(c) *Chromaflex chromatographic column.*—14.5 mm id × 250 mm long with Teflon stopcock (Kontes Glass Co., No. K-420530-222). Glass-blow a 19/22 joint to top of column if possible.

(d) *Evaporative concentrator.*—Kuderna–Danish (K–D) 250 mL; concentrator tubes, 10 mL and 4 mL; Snyder (3-section) and micro Snyder distilling columns (Kontes Glass Co.).

(e) *Tamping rod.*—Glass, 450 mm long with 12 mm diameter disk.

(f) *Gas chromatograph–thermal energy analyzer (GC–TEA).*—Varian Aerograph gas chromatograph Model 2700, or equivalent, interfaced with thermal energy analyzer Model 502. Conditions: 1.8 m × 3.2 mm stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas-Chrom P; helium carrier gas, 40 mL/min; column temperature programmed from 140 to 190°C at 4°/min; injector, 180°C; TEA furnace, 450°C; TEA vacuum, 1.5 mm; liquid nitrogen–ethanol cold trap.

### *Reagents*

(a) *Celite 545.*—Not acid-washed (Fisher Scientific Co.).

(b) *Dichloromethane (DCM), n-pentane and n-hexane.*—Burdick & Jackson Distilled-in-Glass solvents.

(c) *Phosphoric acid.*—6N. Extract once with an equal volume of DCM before use, to remove impurities.

(d) *Sodium sulfate.*—Anhydrous, granular.

(e) *Alumina.*—Basic (Camag); dry in vacuum oven at 140°C for 6 h, and then deactivate with 1.5% water. Store in tightly closed bottle until needed.

(f) *Alundum.*—A. H. Thomas "Boiling stones," 8–14 mesh.

(g) *N-Nitrosothiomorpholine (NTMOR) and N-nitrosoazetidine (NAZET) internal standard.*—Each 0.10 μg/mL DCM.

(h) *N-Nitrosothiazolidine (NTHZ) and NTMOR working standard.*—Each 0.10 μg/mL DCM.

(i) *NPYR and NAZET working standard.*—Each 0.10 μg/mL DCM.

NTHZ, NTMOR, NPYR, and NAZET were synthesized from their corresponding amines and sodium nitrite and purified by fractional vacuum distillation according to the general procedure published previously (5).

### *Sample Analysis*

Weigh 10 g Celite into 250 mL beaker. Add 10 mL 6N $H_3PO_4$, ca 3 mL at a time, and stir Celite with small glass rod until mixture is fluffy and uniform in texture. Using powder funnel, pour resultant acid–Celite mixture into 32 mm id chromatographic column containing glass wool plug at bottom. Insert tamping rod through Celite and tamp from bottom up to achieve a height of ca 25 mm. Accurately weigh 10.0 ± 0.1 g doubly ground fried bacon (passed twice through a 1/8 in. plate) and quantitatively transfer sample to mortar. Add 1.0 mL NAZET–NTMOR internal standard solution (equivalent to 10 ppb) to bacon sample, using 1.0 mL transfer pipet, and then add 25 g sodium sulfate; mix with pestle ca 30 s. Add 20 g Celite to mortar and grind 15–20 s until Celite is thoroughly mixed with sodium sulfate and bacon. Grind with moderate pressure for additional 2 min. Quantitatively transfer free-flowing dry mixture into chromatographic column, and tamp with tamping rod to achieve a total height of ca 100 mm. Add 30 g sodium sulfate to top of column. Rinse mortar and pestle with 10 mL previously prepared pentane–DCM (95 + 5) solvent mixture, and add rinse to column, immediately followed by 90 mL of the same solvent. Begin collection of eluate in 100 mL graduated cylinder. When level of solvent in column just touches top of sodium sulfate, add 125 mL DCM at one time. After 85 mL eluate (Fraction 1) has been collected, change receivers. Collect remaining eluate (Fraction 2) in 250 mL K–D flask and concentrate to 1.0 mL for NPYR–NAZET analysis as described previously (3).

Add 3.0 g alumina to Chromaflex column containing glass wool plug and 15 mL hexane. Add 2.0 g anhydrous sodium sulfate to top of column, and drain hexane to within 25 mm of sodium sulfate. Transfer the 85 mL of eluate (Fraction 1) with 15 mL DCM to 250 mL K–D flask equipped with a 10 mL concentrator tube.

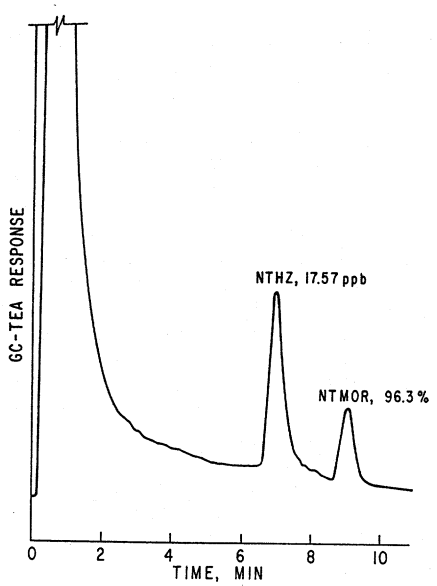


**Figure 1. GC-TEA chromatogram from fried bacon extract, dual column method.**

Add 2 small boiling stones, attach 3-section Snyder column, and concentrate eluate to 6–8 mL on steam bath (sample generally will not concentrate to less than 6 mL because it contains lipid material from the fried bacon). Dilute sample with 2 mL hexane and quantitatively transfer sample to column, using disposable pipet. Rinse concentrator tube twice with 4 mL hexane and add rinses to column. Collect wash eluate in 125 mL Erlenmeyer flask at rate of 2 mL/min. Add an additional 25 mL hexane to column just as solvent reaches top of sodium sulfate layer. When level of solvent in column again just touches top of sodium sulfate, stop flow and add 125 mL DCM to column. Change receivers, discard first eluate, and collect remaining eluate at flow rate of 2 mL/min in 250 mL K–D flask equipped with 4 mL concentrator tube. Concentrate sample as described previously (3) for determination of NTHZ–NTMOR.

## Experimental

The procedure and necessary calculation for determining NPYR and NAZET (Fraction 2) have been described previously (3). NTHZ and NTMOR were determined in a similar manner. Figure 1 shows a typical chromatogram containing NTHZ and the NTMOR internal standard. This method has a minimum detectable level of NTHZ on the GC-TEA system of 1.0 ppb.

**Table 1. Statistical analysis for repeatability of *N*-nitrosothiazolidine method**

| Source of variation | df | Uncorrected NTHZ SS | Uncorrected NTHZ MS | Uncorrected NTHZ F | Corrected NTHZ SS | Corrected NTHZ MS | Corrected NTHZ F | Recovery, % NTMOR SS | Recovery, % NTMOR MS | Recovery, % NTMOR F |
|---|---|---|---|---|---|---|---|---|---|---|
| *N*-Nitrosothiazolidine | 5 | 1045.58 | 209.12 | 38.72** | 1362.70 | 272.54 | 150.06** | 1058.68 | 211.74 | 24.75** |
| Error | 12 | 64.81 | 5.40 | | 21.79 | 1.82 | | 436.77 | 36.40 | |
| Total | 17 | 1110.50 | — | | 1384.49 | — | | 1495.45 | — | |
| Repeatability[a] | | | 2.32 ppb | | | 1.35 ppb | | | 6.03% | |

** $P = <0.01$.
[a] Repeatability = $\sqrt{MS_{error}}$

The procedure used for the mass spectral confirmation of NTHZ in fried bacon extracts was the same as reported previously (1).

Analysis of variance was performed on the measured nitrosamine, according to method described by Snedecor and Cochran (6). Where only the statistical summary is presented, the raw data are available on request. The corrected NTHZ data were adjusted for the recovery of the internal nitrosamine standard.

## Results and Discussion

This dual column chromatographic method, hereafter referred to as the "dual column" method, allows for the rapid analysis of the volatile nitrosamines *N*-nitrosodimethylamine and NPYR and the less volatile NTHZ from the same 10 g bacon sample, when the gas chromatograph is temperature programmed. Because NTHZ elutes in the lipid phase, we used a second internal nitrosamine. NTMOR was selected since it had structural and retention characteristics similar to NTHZ, and the possibility of this nitrosamine being naturally present or formed in fried bacon was considered unlikely. In addition, a highly significant correlation ($r^2 = 0.897$, $P < 0.01$) between NTMOR and NTHZ recovery rates was observed in nitrosamine-free fried bacon fortified at the 10 ppb level.

Six samples of fried bacon were analyzed in triplicate to determine the within-laboratory repeatability of this dual column method. Determinations of NTHZ ranged from 9.90 to 34.94 ppb, corrected (7.73 to 34.49 ppb, uncorrected) and recovery of the NTMOR internal standard ranged from 70.9 to 101.3% with a mean of 93.3%. Analysis of variance of the results (Table 1) indicated that the standard deviation of repeatability of NTHZ determination was 1.35 ppb (CV = 6.35%; 2.32 ppb uncorrected) and repeatability of recovery of the NTMOR standard was 6.03% (CV = 6.46%).

NTHZ results for 16 different samples of fried, cure-pumped bacon, which were analyzed by both the dual column and mineral oil methods, are shown in Table 2. The dual column method gave values that ranged from <1 to 26 ppb with an average of 86% lower than the mineral oil method. These results suggest that NTHZ is produced during analysis by the mineral oil method similar to that reported for NPYR (3). To demonstrate that NTHZ is artifactually formed during mineral oil analysis, 3 commercially cured bacon samples containing normally incurred NTHZ were analyzed with the addition of sodium ascorbate and/or $\alpha$-tocopherol. These compounds are reductants known to destroy nitrite and inhibit nitrosamine formation. The results (Table 3) show that NTHZ formation can be reduced by the addition of inhibitors, but that the NTHZ values are still significantly higher

**Table 2. *N*-Nitrosothiazolidine in fried bacon determined by mineral oil and dual column methods**

| Sample | Nitrosothiazolidine, ppb[a] Mineral oil | Nitrosothiazolidine, ppb[a] Dual column |
|---|---|---|
| 1 | 27.91 | 10.51 |
| 2 | 11.77 | 4.79 |
| 3 | 26.54 | 12.24 |
| 4 | 91.40 | 8.04 |
| 5 | 42.00 | 14.75 |
| 6 | 184.71 | 18.93 |
| 7 | 41.92 | 5.22 |
| 8 | 29.00 | 8.10 |
| 9 | 65.99 | 17.41 |
| 10 | 69.70 | 8.52 |
| 11 | 57.72 | 7.50 |
| 12 | 26.66 | ND[b] |
| 13 | 282.77 | 16.93 |
| 14 | 28.04 | 3.82 |
| 15 | 25.65 | 11.39[c] |
| 16 | 197.65 | 26.20[c] |

[a] Corrected values, using NTMOR internal standard.
[b] None detected.
[c] Confirmed by mass spectrometry.

Table 3. *N*-Nitrosothiazolidine inhibition during mineral oil distillation procedure

| Inhibitors | Nitrosothiazolidine, ppb[a] | | |
|---|---|---|---|
| | Sample 1 | Sample 2 | Sample 3 |
| None | 57.72 | 26.66 | 282.77 |
| NaAsc[b] | 27.73 | 21.31 | 81.65 |
| α-Toc[c] | 19.00 | 14.79 | 89.41 |
| NaAsc + α-toc | 17.68 | 10.89 | 44.91 |
| Dual column | 7.50 | ND[d] | 16.93 |

[a] Corrected for recovery of NTMOR internal standard.
[b] NaAsc: 1500 ppm (aqueous).
[c] α-Toc: 500 ppm (in mineral oil).
[d] None detected.

Table 4. Analysis of variance for intralaboratory study

| Variation | df | SS | MS | F |
|---|---|---|---|---|
| Sample | 4 | 2934.9 | 733.7 | 328.30** |
| Analyst | 2 | 15.8 | 7.9 | 3.54 |
| Analyst × sample | 8 | 17.9 | 2.2 | 1.54 |
| Error | 15 | 21.7 | 1.4 | |
| Total | 29 | 2990.3 | | |

** $P < 0.01$.

than the dual column method results. Apparently, the rate of nitrosation of the precursor amine to NTHZ is sufficiently great compared with the competitive reaction of nitrite and reductant to form nitric oxide, which is not considered a good nitrosating agent; otherwise, little or no NTHZ would be observed. This indirectly suggests that thiazolidine, a weak base, is the precursor. When these same inhibitors were added to the dual column method, there was no significant change in the NTHZ results, which again indicates that the NTHZ is not being artifactually produced during analysis. Also, the results from a previous experiment (1), where either nitrite and/or cysteamine, a thiazolidine precursor, or thiazolidine itself was added to nitrite-free bacon, show that NTHZ can indeed be artifactually formed as a result of mineral oil analysis.

Finally, a limited intralaboratory study using the dual column method was conducted on fried bacon containing normally incurred NTHZ. The samples were analyzed in duplicate by 3 analysts operating independently. The analysis of variance of the corrected results is shown in Table 4. A significant ($P < 0.01$) difference between the bacon samples was observed with an $F$-test, as expected, because fried bacon with a wide NTHZ range was observed (2.92–36.60 ppb). No significant analyst effect or analyst × sample interaction was indicated by the analysis of variance. The standard deviations for repeatability and reproducibility were determined as prescribed by Steiner (7) to be 1.20 (CV = 6.64%) and 1.55 ppb (CV = 8.57%), respectively.

The availability of this method will permit the investigation of how NTHZ is formed, so that procedures for its elimination or reduction can be developed.

## Acknowledgments

The authors thank Robert Gates and Judith Pascale Foster for their technical assistance, and the National Cancer Institute for the loan of a thermal energy analyzer under contract No. NO1-CP-55715.

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fold with respect to *B. licheniformis*. A longer process would provide an added safeguard against the possibility of metabiosis resulting in growth and toxigenesis by *C. botulinum* and would result in reduced losses due to spoilage.

In summary *B. licheniformis* could elevate the pH and deplete oxygen in an aerobically incubated acidic model system, thereby allowing growth and toxin production by *C. botulinum*. Canner size had a large effect on the lethality of a boiling water bath process to *B. licheniformis*. Even if there is no botulinic threat from tomatoes processed by the current recommendations, consideration should be given to the adoption of a longer process which should result in reduced losses due to spoilage. The exact specifications of a longer process should be the subject of further study.


## ACKNOWLEDGMENTS

I thank G. M. Sapers for encouragement and helpful discussions during this study, L. Conway, G. Maher, and A. DiVito for valuable technical assistance, and D. D. Bills, J. L. Smith, and C. N. Huhtanen for helpful comments on the manuscript.


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